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NEGATIVE AMES TESTS OF EPOXIDE FATTY METHYL ESTERS DERIVED FROM HOMOLYSIS OF LINOLEIC ACID HYDROPEROXIDES*

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Abstract Five isomeric epoxyhydroxyene and epoxyoxoene fatty esters derived from homolytic decomposition of linoleic acid hydroperoxide were tested for mutagenicity by the 'Ames' top-agar incorporation method using S-9 mix derived from livers of male rats pretreated with Aroclor 1254. The epoxide fatty esters tested – methyl *trans*-12,13-epoxy-*erythro*-11-hydroxy-*cis*(*trans*)-9-octadecenoate and methyl *trans*-12,13-epoxy-*threo*-11-hydroxy-*cis*(*trans*)-9-octadecenoate (each composed of approximately 80% *cis*-9-ene and 20% *trans*-9-ene), methyl *trans*-12,13-epoxy-9-oxo-*trans*-10-octadecenoate, methyl *trans*-12,13-epoxy-9-hydroxy-*trans*-10-octadecenoate and methyl *cis*-12,13-epoxy-9-oxo-*trans*-10-octadecenoate – had structural characteristics similar to certain potent mutagens. However, these esters were not mutagenic in *Salmonella typhimurium* strains TA100, TA98 or TA1537 at concentrations up to 2000 μg/test plate. Under the same test conditions, the methyl ester of hydroperoxy linoleic acid, from which these epoxides were derived, was weakly mutagenic in strain TA100 and possibly also in strain TA98.

INTRODUCTION

Epoxides are produced as secondary products of lipid oxidation, and some of these fatty epoxides are vicinal to hydroxyl and olefinic groups. Such highly substituted fatty epoxides comprise an important group of compounds found in autoxidation mixtures as well as in biological systems (Gardner, 1980). Epoxides as a class are potential alkylating agents and some are known to be mutagenic (Andersen, Kiel, Larsen & Maxild, 1978; Greene, Friedman, Sherrod & Salerno, 1979; Ortiz de Montellano & Boparai, 1978; Thompson, Coppinger, Piper *et al.* 1981; Voogd, van der Stel & Jacobs, 1981; Wade, Airy & Sinsheimer, 1978; Wade, Moyer & Hine, 1979). The possibility that fatty epoxides may also be mutagenic has been suggested by Mead (1980). Mutagenic epoxides are often activated in a way that facilitates reaction with a base-pair residue of DNA, usually through nucleophilic attack on the epoxide. Among the known examples of such transformations are reactions of the guanine base pair with benzo[*a*]pyrene-7,8-dihydrodiol-9,10-oxide (Jeffrey, Jennette, Blobstein *et al.* 1976b), with 7,12-dimethylbenz[*a*]anthracene-5,6-oxide (Jeffrey, Blobstein, Weinstein *et al.* 1976a) and with the putative 2,3-oxide of aflatoxin B_1 arising from metabolic activation of aflatoxin B_1 (Essigmann, Croy, Nadzan *et al.* 1977). Such mutagenic epoxides are characterized by vicinal substituent group(s), which presumably cause the epoxide to be electron deficient and thus more susceptible to nucleophilic attack.

In this communication we demonstrate that several epoxides derived from the decomposition of linoleic acid hydroperoxide are not mutagenic as determined by the method described by Ames, McCann & Yamasaki (1975).

EXPERIMENTAL

Preparation of epoxide fatty esters. Epoxide fatty esters were produced by radical decomposition of 13-hydroperoxy-*cis*-9-*trans*-11-octadecadienoic acid (linoleic acid hydroperoxide) followed by methyl esterification of the epoxide fatty acid formed. The mechanisms of formation of these compounds are discussed in detail elsewhere (Gardner & Jursinic, 1981; Gardner & Kleiman, 1981). Two separate procedures were used to produce the fatty ester epoxides and five of the major products were isolated for mutagenicity testing.

In the first preparation procedure, 470 mg 13-hydroperoxy-*cis*-9-*trans*-11-octadecadienoic acid (99 + %

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Abbreviations: DMSO = Dimethylsulphoxide; HPLC = high-pressure liquid chromatography; NMR = nuclear magnetic resonance; TLC = thin-layer chromatography.

pure) was prepared by soya lipoxygenase (EC 1.13.11.12) oxidation of linoleic acid (Gardner, 1975) for homolytic decomposition with a cysteine-$FeCl_3$ catalyst (Gardner & Jursinic, 1981). The following isomeric epoxide fatty methyl esters with the epoxide located exclusively at the 12,13-carbons (Fig. 1) were isolated as described by Gardner & Kleiman (1981): methyl *trans*-12,13-epoxy-*erythro*-11-hydroxy-*cis* (*trans*)-9-octadecenoate (I), methyl *trans*-12,13-epoxy-*threo*-11-hydroxy-*cis* (*trans*)-9-octadecenoate (II), methyl *trans*-12,13-epoxy-9-oxo-*trans*-10-octadecenoate (III), methyl *trans*-12,13-epoxy-9-hydroxy-*trans*-10-octadecenoate (IV) and methyl *cis*-12,13-epoxy-9-oxo-*trans*-10-octadecenoate (V). Compounds I and II were approximately 80% *cis*-9-ene and 20% *trans*-9-ene. Because product IV was largely lost by solvolysis, this compound was obtained by reduction of a portion of I by $NaBH_4$ in methanol at 0 C. The reduced ester was extracted from the reaction mixture into $CHCl_3$ by addition of $CHCl_3$ and water to form the mixture $CHCl_3$ CH_3OH–H_2O, 2:1:1 (by vol.). Preparative TLC using hexane–ether, 1:1 (v/v) was used to purify the reduced ester. The purity of the isolated esters was assessed by analytical TLC, and the identity was confirmed by NMR and optical rotatory dispersion.

With the second isolation procedure, larger quantities of epoxide esters were obtained from 1.9 g linoleic acid hydroperoxide, as described by Gardner & Crawford (1981). Since the hydroperoxide was composed of a mixture of 85% 13-hydroperoxy-9,11-octadecadienoic acid and 15% 9-hydroperoxy-10,12-octadecadienoic acid, the epoxide position of the esterified products reflected the same ratio. Thus compound III consisted of 85% methyl *trans*-12,13-epoxy-9-oxo-*trans*-10-octadecenoate and 15% methyl *trans*-9,10-epoxy-13-oxo-*trans*-11-octadecenoate. These positional isomers, which differ only in the way the functional groups are situated in relation to the fatty acid carbon chain, are so similar in their chemical and physical properties that they were not completely separable by chromatography. Therefore each fatty ester epoxide was isolated as an 85:15 mixture. The esters prepared in this way were purified by column chromatography followed by HPLC, using the procedure previously reported by Gardner & Crawford (1981). Portions (15–20 mg) of epoxyhydroxyene and epoxyoxoene esters were purified by HPLC with 6 and 4% acetone in hexane, respectively. After HPLC separation, the isolates were washed according to the procedure of Folch, Lees & Sloane Stanley (1957). Compound IV was prepared as described above. Fractions that contained minor impurities, as assessed by analytical TLC, were purified further on preparative TLC plates developed in hexane–ether, 3:2 (v/v).

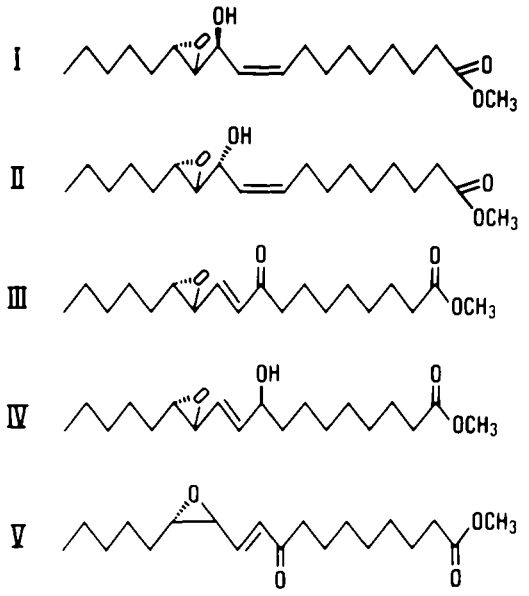

Fig. 1. Structures of fatty ester epoxides tested: (I) methyl *trans*-12,13-epoxy-*erythro*-11-hydroxy-*cis*(*trans*)-9-octadecenoate; (II) methyl *trans*-12,13-epoxy-*threo*-11-hydroxy-*cis*-(*trans*)-9-octadecenoate; (III) methyl *trans*-12,13-epoxy-9-oxo-*trans*-10-octadecenoate; (IV) methyl trans-12,13-epoxy-9-hydroxy-*trans*-10-octadecenoate; (V) methyl *cis*-12,13-epoxy-9-oxo-*trans*-10-octadecenoate. Compounds I and II were each approximately 80% *cis*-9-ene and 20% *trans*-9-ene (the latter structure is not shown). Because the epoxides were derived from homolytic decomposition of the soya lipoxygenase-produced 13-hydroperoxide of linoleic acid, the 13-carbon of the epoxide was chiral as shown.

Mutagenicity tests. Quantitative plate tests using the top agar overlay technique described by Ames *et al.* (1975) were used. Three histidine-dependent mutants of *Salmonella typhimurium* (TA100, TA98 and TA1537), obtained from Professor B. N. Ames, served as test strains. The S-9 microsomal fraction was prepared from Aroclor 1254-induced male rat liver as described by Ames *et al.* (1975) and was used at a level of 100 μl S-9/ml of S-9 mix. Test materials (compounds I–V above and the methyl ester of 13-hydroperoxy-*cis*-9-*trans*-11-octadecadienoic acid) were dissolved in dimethylsulphoxide (DMSO) and were added to top agar in a volume of 0.1 ml. Revertants obtained were confirmed by subculturing on histidine-free media.

Stability study. To confirm the stability of the epoxide fatty esters in DMSO, unused portions of the DMSO solutions used for the mutagenicity tests were stored at −10 C for 2 months and then extracted for analysis. One part DMSO solution was added to 20 parts $CHCl_3$–CH_3OH–H_2O, 2:1:1 (by vol.) and the $CHCl_3$ layer containing the test epoxide was washed three times with water prior to analysis by TLC and NMR. The epoxide fatty esters recovered in this way were found to be unchanged.

RESULTS

The epoxide fatty esters were not mutagenic to the histidine-dependent *Salmonella typhimurium* strains used in the test (Table 1). The number of revertants per plate was not significantly greater than the control even when the epoxide fatty esters were tested at the 2000-μg level. Since there were no significant differences in the results regardless of the method used to obtain the fatty esters, test results obtained with compounds from both of the isolation procedures are included in Table 1. The pure positional isomers were tested up to the 1000-μg/plate level, except for compounds I and V which were assessed up to 500 μg/plate. The material isolated from the large-scale prep-

Table 1. Mutagenicity tests of methyl esters of epoxide fatty acids using *Salmonella typhimurium* strains TA100, TA98 and TA1537

Test compound† and concn (μg plate)	No. of revertants plate													
	TA100						TA98				TA1537			
	− S-9			+ S-9			− S-9		+ S-9		− S-9		+ S-9	
I 2000			109			152		27		32		5		5
500	162*	130	110		120	137	29	33	46	41	4	4	4	8
50	120													
5	109													
0‡	114	133	124		129	140	38	27	44	45	8	6	12	7
PC§	623**				1725**	1450**			1915**	1825**	630**	3800**		
II 2000			133			112		19		29		6		68
1000	130	100			122		18		41		5		10	
500			88			124		30		28		7		6
100	160													
10	134													
0‡	(114)	(133)	(124)		(129)	(140)	(38)	(27)	(44)	(45)	(8)	(6)	(12)	(7)
PC§	(623)**				(1725)**	(1450)**			(1915)**	(1825)**	(630)**	(3800)**		
III 2000			75			98		14		27		3		2
1000	171	188		135			*32*		*48*		*16*		*20*	
500			88			117		18		29		5		7
100	*120*	126		*124*			*25*		*44*		*10*		*13*	
10	*155*	142		*162*			*24*		*44*		*15*		*9*	
0‡	176	154	(124)	147		(140)	29	(27)	43	(45)	11	(6)	13	(7)
PC§		2500**		1940**		(1450)**			1935**	(1825)**	1975**	(3800)**		
IV 2000			72¶			96¶		20		33		3¶		6¶
1000	173	125		160			*36*		*36*		*11*		*10*	
500			116			136		20		33		4¶		11¶
100	*143*	125		*144*			*26*		*38*		*14*		*14*	
10	*182*	150		*167*			*26*		*51*		*10*		*13*	
0‡	(176)	(154)	(124)	(147)		(140)	(29)	(27)	(43)	(45)	(11)	(6)	(13)	(7)
PC§		(2500)**		(1940)**		(1450)**			(1935)**	(1825)**	(1975)**	(3800)**		
V 2000			137			146		21		35		3		5
500	117	130	111		135	162	36	27	45	48	11	9	10	8
50	115													
5	125													
0‡	(114)	(133)	(124)		(129)	(140)	(38)	(27)	(44)	(45)	(8)	(6)	(12)	(7)
PC§	(623)**				(1725)**	(1450)**			(1915)**	(1825)**	(630)**	(3800)**		

†Compounds: I = *trans*-12,13-epoxy-(*erythro*-11-hydroxy-(*cis*/*trans*)-9-octadecenoate; II = *trans*-12,13-epoxy-(*threo*-11-hydroxy-*cis*(*trans*)-9-octadecenoate; III = *trans*-12,13-epoxy-9-oxo-*trans*-10-octadecenoate; IV = *trans*-12,13-epoxy-9-hydroxy-*trans*-10-octadecenoate; V = *cis*-12,13-epoxy-9-oxo-*trans*-10-octadecenoate.

‡Negative controls: 0·1 ml dimethylsulphoxide/plate.

§Positive controls (PC) were: for strains TA100 and TA98: − S-9, aflatoxin B_1 at 0·25 or 0·5 μg/plate; for TA100: + S-9, 4-nitroquinoline-*N*-oxide at 0·05 or 0·1 μg/plate; for TA1537: − S-9, 9-aminoacridine at 100 μg/plate.

Colonies were not histidine-independent when subcultured and are therefore not true revertants.

¶Growth inhibition of background lawn.

Values for all negative and positive controls and, where italicized, for test compounds are means of duplicate determinations; other values for test compounds are single plate determinations. Tabulation of more than one value for one assay indicates that separate experiments were conducted. Experiments were grouped so that positive controls for both strain and S-9 activation were included in each. Control values are given in parenthesis when they were repeated for compounds tested in groups.

Single asterisks mark values exceeding the concurrent control by more than 1‴ confidence level least significant difference ($LSD_{0\cdot01}$): $LSD_{0\cdot01} = t_{0\cdot01}\ ms$ (where t = t statistic with df equal to the number of control data pairs; ms = overall variance of individual replicate control differences from the mean of each control pair, summed over all control pairs). Double asterisks mark values differing by more than $LSD_{0\cdot001}$. *$LSD_{0\cdot01}$ levels: TA100: − S-9, 46 (n = 7); TA100: + S-9, 67 (n = 4); TA98: − S-9, 17·6 (n = 4); TA98: + S-9, 38 (n = 4); TA1537: − S-9, 20 (n = 4); TA1537: + S-9, 10 (n = 4). **$LSD_{0\cdot001}$ levels: TA100: − S-9, 71·5 (n = 7); TA100: + S-9, 125 (n = 4); TA98: + S-9, 71 (n = 4).

aration, which contained the mixed positional isomers, was used for the 2000-μg/plate tests as well as for the lower levels.

At the higher dose levels, the growth of the background lawn that subsists on the traces of histidine included in the media was inhibited in some cases, as indicated in Tables 1 and 2. Methyl *trans*-12,13-epoxy-9-hydroxy-*trans*-10-octadecenoate and methyl 13-hydroperoxy-*cis*-9,*trans*-11-octadecadienoate were the most inhibitory. Of the five epoxy esters tested, the 12,13-epoxy-9-hydroxy-10-ene fatty ester (IV) was the most susceptible to solvolysis and NIH shift reactions of the epoxide (Gardner & Kleiman, 1979).

In contrast to the results with the epoxy fatty esters, the hydroperoxide itself, methyl 13-hydroperoxy-*cis*-9,*trans*-11-octadecadienoate, was not only toxic to Salmonella but also weakly mutagenic (Table 2).

Although the epoxide fatty esters in this study were readily solubilized by DMSO and did not appear to separate into globules in the overlay, we tested the esters after sonication with Tween 20 according to Yamaguchi & Yamashita (1979). The results we obtained after sonication (data not shown) were similar to results using DMSO solubilization. On the other hand, methyl 13-hydroperoxy-*cis*-9,*trans*-11-octadecadienoate did form small globules within the agar overlay when DMSO solutions were used at the higher dose levels. However, sonication of the hydroperoxide with Tween 20 did not yield reversion rates significantly different from those obtained by application of the sample dissolved in DMSO.

DISCUSSION

Although epoxides are known alkylating agents and many are potent mutagens, the lack of mutagenic effect with the epoxide fatty esters tested in this study is not inconsistent with previous published reports on structurally related compounds. These epoxy fatty esters possess two structural features reported to be associated with lack of mutagenicity. The first is 1,2-disubstitution, which generally results in markedly reduced mutagenicity compared with that of compounds with the epoxy group in the ω position of an alkyl chain (Ivie, MacGregor & Hammock, 1980; Voogd *et al.* 1981; Wade *et al.* 1978). The epoxide fatty esters studied here belong to this class of hindered epoxide. When mutagenicity of 1,2-disubstituted compounds has been reported, the substituent groups have generally been strongly electron-withdrawing. Secondly, systematic studies of mutagenically active epoxides of increasing chain length indicate that mutagenic activity drops dramatically when the carbon chain exceeds 4–6 carbons (Thompson *et al.* 1981; Voogd *et al.* 1981), presumably because of factors such as solubility and cell penetration. This effect of chain length appears to affect genetic activity similarly both in Salmonella and in mammalian, including human, cells (Thompson *et al.* 1981). Our results, while not constituting a truly comprehensive study of the potential genotoxicity of fatty epoxides, suggest that the genotoxic potential of these compounds may be minimal. The reactivity of compound IV (the 12,13-epoxy-9-hydroxy-10-ene fatty ester), as evidenced by its susceptibility to solvolysis, presumably stems from the instability of the allylic epoxide.

Table 2. Mutagenic activity of methyl 13-hydroperoxy-*cis*-9,*trans*-11-octadecadienoate

Concn (μg plate)	No. of revertants per plate											
	TA100				TA98				TA1537			
	S-9		+S-9		−S-9		+S-9		−S-9		+S-9	
1000		0†										
500	538**†‡		300**	**182**†**	55†	0†	41†	94*	0†	13†	0†	13†
100		233**	164	138	35	37	44	58	2†	19†	8	16
50	*164*		158	148	39	26	41	58	4	8	7	11
10	*111*											
0§	132	114	171	133	38	27	44	46	9	8	12	12
PC		623**			1725**	1075**	1915**	1175**	3485**	630**		

†Growth inhibition of background lawn, occurring mainly adjacent to droplets of undissolved test compound which formed at the higher dose levels.

‡This point (only) was 606 μg plate. Duplicate plates were run: in one case the sample in 0.18 ml ether was evaporated to dryness in the top agar tube and immediately taken up in 0.1 ml DMSO; in the second a small amount of ether was allowed to remain. The individual values were 515 and 560 revertants plate, ruling out decomposition during ether removal as a cause for the positive response. 100 μl ether did not affect the spontaneous revertant frequency. Revertants were confirmed by restreaking on histidine-free agar.

§Negative controls: 0.1 ml dimethylsulphoxide plate.

Positive controls (see footnote ¶, Table 1).

Values are means of duplicate determinations except where italicized (single plate only) or in bold print (four plates). Tabulation of more than one value for one assay indicates that separate experiments were conducted. Values marked with asterisks differ significantly from the concurrent control: *$LSD_{0.01}$; **$LSD_{0.001}$ (see footnote to Table 1).

Because of the susceptibility of this epoxide to nucleophilic attack, it would probably be the most highly suspect as a mutagen and, particularly in view of the toxicity observed, further studies of this compound in other test systems would be of interest.

Unlike the epoxide esters tested, methyl linoleate hydroperoxide exhibited weak mutagenicity under the conditions we used. Lipid hydroperoxides are the primary products of lipid oxidation, and in turn the precursors of fatty epoxides. Recently, Yamaguchi & Yamashita (1980) also obtained a weak mutagenic response with isolated hydroperoxides of methyl linoleate and linolenate using the method of Ames *et al.* (1975). It cannot be established with certainty whether these results stemmed from the hydroperoxide itself or a decomposition product generated during the course of incubation with Salmonella. However two lines of evidence suggested that the response was due to the hydroperoxide group rather than to secondary products of hydroperoxide decomposition: (a) a similar mutagenic response was observed with two other hydroperoxides, *tert*-butylhydroperoxide and cumene hydroperoxide, but not with peroxides, peracids and H_2O_2 (Yamaguchi & Yamashita, 1980); (b) the mutagenic effect of autoxidized linolenic acid correlated with the peroxide value of the mixture (Yamaguchi & Yamashita, 1979). It is, of course, possible that both these results and our own could be due to decomposition products generated during the course of the mutagenicity assay. On the other hand, Scheutwinkel-Reich, Ingerowski & Stan (1980) were unable to observe mutagenic activity for linoleic acid hydroperoxide. These workers did not use the same procedures for solubilizing the hydroperoxide as were used by Yamaguchi & Yamashita (1979), whose study indicated that the method of solubilizing test lipids could affect the reversion rate of Salmonella strains. They reported that autoxidized linolenic acid did not elicit increased reversion rates unless the fatty acid was sonicated with either detergent or protein. However, we were able to observe a weak response without the use of special solubilization techniques. It is noteworthy also that linoleate hydroperoxides have been reported to have both toxic effects (Holman & Greenberg, 1958) and reproductive effects (Cutler & Schneider, 1973) in test animals.

While the mutagenicity of lipid hydroperoxides may be due to initiation of free radical chains, which then cause formation of DNA radicals (Fukuzumi, 1978; Pietronigro, Seligman, Jones & Demopoulos, 1976), the possibility that fatty hydroperoxides may initiate free-radical oxidation of endogenously present carcinogens to the ultimate "active" form is suggested by the results of Floyd, Soong, Walker & Stuart (1976) and Dix & Marnett (1981). For example, the latter authors have shown that the radical reactions leading to fatty epoxide formation may be responsible for activation of 7,8-dihydroxy-7,8-dihydrobenzo[*a*]-pyrene to the mutagenic 7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene. Although fatty epoxides are also among the end products by this mechanism, they probably have no mutagenic role *per se*. Inasmuch as lipid hydroperoxides may cause carcinogen activation, this illustrates the necessity of considering indirect pathways that may activate secondary cellular constituents to a mutagenic derivative. Whether lipid oxidation products play a causal role in cancer initiation (or promotion) cannot be answered without much additional research.

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